COMMENTARY

National Cancer Institute Workshop Report: The Phakomatoses Revisited

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A workshop, "The Phakomatoses Revisited," sponsored by the Office of Rare Diseases of the National Institutes of Health (NIH) and the Division of Cancer Epidemiology and Genetics of the National Cancer Institute (NCI), was held in Rockville, MD, March 2–3, 1999. Motivation for the workshop came from the cloning of the genes for 10 dominantly inherited disorders that are classified as phakomatoses: neurofibromatoses 1 and 2 (NF1 and NF2, respectively), tuberous scleroses 1 and 2 (TSC1 and TSC2, respectively), von Hippel–Lindau disease (VHL), nevoid basal cell carcinoma syndrome (NBCCS), Cowden disease (CD), Peutz–Jeghers syndrome (PJ), juvenile polyposis (JP), and familial adenomatous polyposis (FAP).

In a clinical medicine and pathology session chaired by M. Tucker (NCI, Bethesda, MD), B. Korf (Children's Hospital, Boston, MA) reviewed the concept of the phakomatoses as proposed by van der Hoeve in 1932. The original concept, which was based on careful clinical and pathologic observations that have been largely ignored in the past decade, focused on scattered congenital spots and tumefactions. The phakomatoses have often been referred to as neurocutaneous conditions because of the frequent involvement of the skin, the eyes, and the nervous system, but other tissues (e.g., bone in NF1 and kidney in TSC) are often affected. Characteristic of the phakomatoses is the patchy or "spotty" manifestations of the abnormalities, which is consistent with a stochastic effect in an individual with an altered tumor suppressor gene.

Korf illustrated the characteristics of phakomatoses by use of NF1, the first to be described and the most common of this group of diseases, as a model. Characteristic signs of NF1 are neurofibromas, café-au-lait spots, skin-fold freckling, and Lisch nodules in the iris. Plexiform neurofibromas are dysplastic lesions with overlying cutaneous hypertrophy; they are regarded as "two-hit" lesions, originating early in life. Optic gliomas are also relatively frequent. Multiple abnormalities are common on magnetic resonance imaging brain scans and may be related to learning disabilities, possibly due to "one-hit" lesions with haploinsufficiency of the NF1 gene. This situation appears to explain why mice heterozygous for the gene have difficulty performing tasks of spatial memory and learning.

M. Wallace (University of Florida, Gainesville) discussed mutation analysis in NF1 disease, in which there is a high mutation rate—50% of affected individuals have new mutations. More than 200 different mutations have been described, with only a few (10–20) recurring. Most are presumed to produce truncated forms of neurofibromin, the gene's protein product, that usually lack a guanosine triphosphatase-activating (GAP) domain. It is unknown if such truncated proteins are stable or have any function. Loss of the domain interferes with signal transduction through the RAS gene. Loss of heterozygosity (LOH) appears more common in plexiform neurofibromas than in dermal neurofibromas. Most NF1 tumors appear to have loss of neurofibromin but not necessarily LOH; some may have subtle second mutations.

D. Gutmann (Washington University, St. Louis, MO) discussed the role of the NF1 gene in developmental biology. Since plexiform neurofibromas are considered by many to be congenital lesions, the initiating events must occur in early development or embryogenesis. The NF1 gene is expressed ubiquitously in embryos but not in adult tissues. Mice that are homozygous for deletion mutations of the gene die of cardiac vessel defects during embryogenesis. In separately cultured Schwann cells and fibroblasts from neurofibromas, the transcript of NF1 is low to absent in the Schwann cells but abundant in the fibroblasts; protein is also abundant in fibroblasts, indicating that the abnormal cell in neurofibromas is the Schwann cell.

There appears to be functional diversity in the NF1 protein with alternative splicing that affects all organs and tissues. The GAP-related domain in NF1 is important for the conversion of RAS–guanosine triphosphate (GTP) complexes to RAS–guanosine diphosphate (GDP) complexes, but the functions of the rest of the protein are not known. Reproducible splice variants have been described in exons 9A, 23A (GAP domain), and 48A. The exon 9A splice variant is developmentally regulated and found only in neurons in the central nervous system (CNS), not in peripheral or enteric neurons or in glial cells; in mice and rats, its expression is increased both *in vivo* and *in vitro* postnatally. The 23A form, which is not as efficient as the 9A variant at inducing hydrolysis of RAS–GTP complexes, is not restricted to any specific tissue, although neurons tend to lack it while glial cells have more of it. The 48A variant is restricted to muscle tissue and may be relevant to the reported excess of rhabdomyosarcoma that is associated with NF1, since excessive expression of the RAS gene blocks the maturation of myoblasts to myocytes.

M. Linehan (NCI, Bethesda) discussed VHL, which is caused by mutations in a classic tumor suppressor gene, VHL. In VHL, kidney tumors have either loss or methylation of the normal allele. Thirty-five percent to 40% of affected individuals die of clear-cell renal cancers, which are usually multifocal (up to 600 tumors and 1100 cysts per kidney). Sporadic clear-cell renal carcinomas frequently also have mutations in VHL. Individuals

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carrying a mutation in the VHL gene develop multiple (frequently extra-adrenal) pheochromocytomas, pancreatic cysts, and solid neuroendocrine tumors. Retinal angiomas are frequent, as are CNS hemangioblastomas. All VHL disease-related tumors are highly vascular; the tumors express vascular endothelial growth factor (VEGF).

When the whole gene is deleted in the germline, there appears to be an attenuated phenotype with a predominant clinical manifestation in the CNS, which may result from poor tolerance of LOH. Exon 3 mutations are especially related to development of pheochromocytomas. Decreased expression of p27, the cyclin-dependent kinase (CDK) inhibitor, in tumors is thought to affect cell cycle regulation.

C. Eng (Ohio State University, Columbus) discussed hamartomas and their relationship to phakomatoses. Hamartomas are benign tumors that are developmentally aberrant but frequently contain all elements of a tissue; the individual cells appear normal. Several disorders, including CD, Bannayan–Riley–Ruvalcaba syndrome (BRR), PJ, TSC, VHL, NF1, NF2, and JP, have hamartomas as prominent clinical manifestations. There is thus a large overlap between hamartomoses and phakomatoses. In general, both have multiorgan involvement. Eng contrasted the extent of involvement of skin, thyroid, breast, genitourinary tract, soft tissues, and brain in CD, BRR, PJ, and JP. In all of these diseases, gastrointestinal hamartomas are a prominent feature.

M. Turner (NCI, Bethesda) made a strong case for including multiple endocrine neoplasia type 1 (MEN1) among the phakomatoses. MEN1 is characterized by parathyroid adenomas, with 96% of affected individuals having hyperparathyroidism; pancreatic adenomas resulting in Zollinger–Ellison syndrome or insulinoma; and anterior pituitary adenomas with prolactinomas, Cushing's disease, or acromegaly. Turner discovered that 88% of MEN1 patients had multiple facial angiofibromas, heretofore considered pathognomonic for tuberous sclerosis. These lesions were clinically and histologically indistinguishable from those found in TSC, although they were generally fewer in number. Their histology showed an increase in plump spindle and stellate cells representing fibroblasts and dermal dendrocytes in the dermis, which also appeared more fibrotic, especially around hair follicles. There was likewise an increase in the number and diameter of the blood vessels. Also noted was a variety of other skin findings, including multiple, small collagenomas in 72% of patients, café-au-lait spots in 38%, lipomas in 34%, and confetti-like hypopigmentation and gingival papules in 6%. The incidence of lipomas is well above the normal background, as are collagenomas. The finding of confetti-like hypopigmentation, gingival papules, and a couple of subungual fibromas is interesting, since such lesions are also seen in TSC. Cutaneous findings can identify individuals at risk of MEN1. Angiofibromas, lipomas, and collagenomas from affected individuals have shown LOH in the MEN1 gene by fluorescent *in situ* hybridization analysis.

Four topics were discussed in the genetics section of the workshop (chaired by A. Goldstein, NCI, Bethesda): mosaicism, new mutations, homozygotes, and one- and two-hit lesions.

Mosaicism is defined as the presence of a mutation, deletion, or chromosomal abnormality in a subpopulation of cells. Mosaicism may be somatic, affecting somatic cells only; gonadal, affecting germ cells only; or gonosomal, affecting a proportion of both types of cells. Detection of low-level mosaicism is possible by analyzing multiple tumors from a single individual. Discovery of identical mutations in two or more different tumors would be a virtually conclusive demonstration of mosaicism.

G. Evans (St. Mary's Hospital, Manchester, U.K.) used NF2 to illustrate the topic of mosaicism. Among 146 classically defined NF2 families, 72 (49%) had identifiable mutations. In contrast, only 10 (6%) of 161 families with variants of the disease had mutations. The mutation detection rate in familial NF2 cases was 20% higher than the rate in nonfamilial cases. In addition, the frequency of affected offspring of index subjects without mutations was reduced as compared with those with mutations. Both factors provided evidence for mosaicism in NF2. In fact, examination of tumor and non-tumor tissue revealed six cases of proven mosaicism. Evans hypothesized that as many as 15% of index subjects might be mosaics. He based his hypothesis on the finding of lower than expected numbers of affected offspring of sporadic classically affected individuals, the low levels of mutation detection, and the differences in the mutation detection frequencies in familial versus nonfamilial cases of the disease.

What is currently known about mosaicism? While many conditions would not manifest themselves if sufficient cells were homozygously normal, tumor-prone conditions may be evident when only a small proportion of cells are affected. Mosaicism is predicted for diseases with high new mutation rates and, as such, is likely to be an important component for many of the phakomatoses. As examples, NF2 and NF1 may both be fully expressed in a mosaic form; NF1 also shows segmental mosaicism. TSC may also appear in mosaic form, although the expression of the disease is usually silent or subtle. Mosaics for VHL and NBCCS have also been observed. Additional research will show the extent and importance of mosaicism in the phakomatoses.

In general, large fractions (one fourth to more than one half) of the phakomatoses are the result of new mutations. E. Henske (Fox Chase Cancer Center, Philadelphia, PA) presented data on TSC1 and TSC2. Comparisons of TSC1 with TSC2 illustrate the complicated nature of such new mutations. The TSC1 gene is located at chromosome 9q34; TSC2 is located at chromosome 16p13. The types of mutations differ for the two diseases. In TSC1, there are essentially no missense mutations; in contrast, for the TSC2 gene, this number is about 20%. Also, the new mutation rate differs between the two forms of TSC; approximately 70% of TSC2 cases appear to have arisen as new mutations versus 40% of new mutation cases for TSC1. These differences may reflect stronger selection against TSC2.

For many of the phakomatoses, the relevant genes are large, and mutations appear throughout the gene. Little evidence for mutational differences is apparent when inherited alterations are compared with new mutations. The characteristics of the genes and their functions likely contribute to the observed types and rates of mutation.

Human homozygotes for the phakomatosis genes have not been reported, but T. Jacks (Massachusetts Institute of Technology, Cambridge) presented results from several mouse models of tumor suppressor genes. Although there are important species-specific differences in tumor development, mouse models may be very useful for understanding embryonic development and homozygous lethality. For example, examination of NF1 homozygous knockout (deletion mutant) mice revealed a lethal cardiac defect mediated by abnormal endocardial cushion cells at mid-gestational age (embryonic days 13.5–14.5). NF2 homo-

zygous knockout mice also have a lethal cardiac phenotype, but one that is mediated by defective extra-embryonic ectoderm that can be detected at embryonic day 7.5.

Homozygous lethal mutations reveal genes that are essential for development. The lethal phenotype illustrates when and where a gene is first required. Even if a gene functions later in life and in different tissues, lethality demonstrates the initial functioning of an essential gene. Thus, a mouse that is a homozygous mutant is a good source of cells for examining protein function. It is essential, however, that the genetic background of the mouse be considered, since it is critical for phenotypic variability and must be taken into account during any gene evaluation. Currently, knockout or mutant mice exist for a number of the phakomatosis genes, including NF1, NF2, VHL, PTCH, TSC2 (Eker rat), APC, PTEN, and SMAD4. The homozygous genotype of each of these genes is lethal.

Several questions exist regarding one- and two-hit lesions. When do second hits occur? Why do they occur? Second hits have been hypothesized to occur because of stochastic processes, environmental exposures, genetic background, or modifying genes, or a combination of these factors.

A. Bale (Yale University, New Haven, CT) illustrated examples of one- and two-hit lesions by use of NBCCS or Gorlin's syndrome. NBCCS includes numerous developmental defects, some of which are symmetric and some that are random. The symmetric defects include generalized overgrowth of the entire body, brain malformations, and coarse facies. Random defects include spinal abnormalities, rib anomalies, and jaw cysts. Odontogenic keratocysts (OKCs) are byproducts of tooth development, likely resulting from abnormal migration or abnormal differentiation of the dental lamina. Microdissected OKCs from NBCCS patients show LOH in the 9q region where the NBCCS gene PTCH is located. The majority of the basal cell carcinomas show allelic loss and are two-hit lesions. Common sporadic basal cell carcinomas are also generally mutant for PTCH. In contrast, a few sporadic OKCs show allelic loss, but most show no evidence of LOH in chromosome 9q, suggesting heterogeneity in OKC etiology.

The PTCH gene, a human homologue of the *Drosophila* patched (ptc) gene, is a key element in the hedgehog (HH) signaling pathway. Sonic HH, the most common vertebrate homologue of *Drosophila* HH, is required for correct patterning of the neural tube and somites and anterior/posterior positioning of the limb bud. The PTCH gene illustrates a phenomenon seen in many of the human phakomatoses: Several genes identified as essential for embryonic development in *Drosophila* are tumor suppressor genes or oncogenes in mammalian cells. The tumors occurring in these disorders result from second hits, although some of the developmental defects may result from haplo-insufficiency. For example, in NBCCS, the symmetric defects (such as overgrowth or coarse facies) are hypothesized to arise as a result of haplo-insufficiency. Experiments in knockout mice are currently under way to test this hypothesis.

In a molecular biology session (chaired by M. Dean, NCI, Frederick, MD), the molecular features of the phakomatoses were examined further. J. DeClue (NCI, Bethesda) discussed the role of RAS and RAS-related genes and their regulation by the NF1 gene product. Activation of RAS protein by binding to GTP is a component of the signal transduction pathway of a number of growth factor receptors and mitogens. The RAS–GTP complex induces the activation of RAF-family proteins, which work through mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEKK) to activate transcription factors and mitogenic target genes. NF1 protein regulates the conversion of RAS–GTP complexes to RAS–GDP complexes and is a negative regulator of the MEKK pathway. In a similar fashion, tuberin, the product of the TSC2 gene, is a negative regulator of the RAS-related RAP1 protein. Consistent with this relationship, sporadic astrocytomas exhibit reduced or absent tuberin expression or elevated RAP1 protein expression.

TSC was discussed further by R. Yeung (University of Washington, Seattle), who discovered that a mutation in TSC2 was responsible for the phenotype of the Eker rat, which displays virtually 100% penetrance for renal cell carcinoma. Rats homozygous for the mutation die in fetal life with CNS abnormalities. The protein products of TSC1 and TSC2, harmartin and tuberin, interact and have Rap1 GAP activity. Cell cycle control is impaired in that homozygously mutant TSC2 cells do not enter G_0, and expression of the p27 protein is repressed.

B. Zbar (NCI, Frederick) discussed the spectrum of mutations in the VHL gene and their relationship to the cancer profiles of VHL disease families. Whereas families that lack pheochromocytoma have VHL gene mutations that include missense and nonsense mutations, 90% of mutations in families with pheochromocytoma are missense mutations. The VHL protein has been demonstrated to interact with the elongin B and C proteins, transcriptional elongation factors. In yeast, however, elongin B and elongin C form a complex that is involved in ubiquitination of proteins targeted for degradation. Expression of VEGF messenger RNA is increased in VHL disease-associated tumors. *In vitro*, serum deprivation causes these cells to undergo apoptosis, whereas wild-type cells enter G_0 and expression of p27 is stimulated.

A wide variety of human tumors display LOH in 10q23, including approximately 70% of glioblastomas and approximately 60% of advanced prostate cancers. This observation suggests that chromosome 10 encodes a tumor suppressor gene. The PTEN (phosphatase and tensin homologue deleted on chromosome 10) gene was isolated from this region and encodes a 403-amino acid protein with a tyrosine phosphatase domain and a large region of homology to tensin and auxilin. Recent data have demonstrated that the principal function of the protein products of the PTEN/MMAC1 gene is the dephosphorylation of the second messenger PIP3. Eng presented data showing that the level of PTEN protein is reduced or absent in a wide variety of hematologic malignancy cell lines, including many in which mutations have not been described. While between 10% and 40% of hematologic malignancies may show somatic PTEN mutations and deletions, as many as 50% of hematologic malignancies have reduced PTEN transcript, and more than 70% have reduced levels of the protein, suggesting that the gene can be inactivated by multiple mechanisms, such as methylation, reduced translation, or increased degradation of the protein.

L. Aaltonen (University of Helsinki, Finland) reviewed the inherited syndromes in which polyps are a notable feature: FAP, PJ, JP, and CD. The PJ syndrome is characterized by hamartomatous polyps in the bowel and an elevated incidence of tumors, with those of the small intestine and the breast being most prominent. Positional cloning led to the isolation of the serine/threonine kinase LKB1 gene, which is expressed in all cell types and shows an expression pattern similar to that of PTEN. To date, LKB1 has not been found to be mutated to a noteworthy

extent in sporadic tumors; however, a mutation has been reported in a sporadic testicular cancer.

The hamartomatous polyps that occur in the JP syndrome in many families appear to be the consequence of germline mutations in the SMAD4/DPC4 gene. However, these hamartomas are histologically distinct from those of PJ, and the risk of malignancy also differs in these two syndromes. It is interesting that several unrelated JP families have the same 4-base-pair deletion, suggesting that this is a frequently recurring mutation. Some JP families display mental retardation, raising the possibility that SMAD4 gene plays a role in neuronal development.

Dean outlined the role of the patched (PTCH) gene in NBCCS. The gene is the human homologue of one from *Drosophila* in which mutations cause abnormal development of a number of adult structures, such as wings and legs. In mice, the HH/PTCH pathway is also active in the developing embryo, affecting the developing spinal cord, gut, and limbs. Numerous tumor suppressor genes and oncogenes are found in this pathway. An intimate connection is noted between genes involved in embryonic development and genes involved in growth control, suggesting that the control of cell growth is a critical component of cellular differentiation.

In a final session (chaired by A. Knudson, Fox Chase Cancer Center), general features of the phakomatoses were discussed. There was agreement that the list of cloned phakomatosis genes justifiably includes NF1, NF2, TSC1, TSC2, VHL, PTCH, PTEN, LKB1, SMAD4, and APC. MEN1 should probably be included also. In each disorder, there are scattered hamartomatous or adenomatous two-hit lesions in one or more organs that can become malignant with low probability. The original definition of phakomatosis is preferred over the later one of a neurocutaneous syndrome. Some phenotypic features may constitute one-hit lesions, i.e., be manifestations of the heterozygous state that are due to haplo-insufficiency or dominant-negative effect. The phenomena of new germline mutation and mosaicism are well known for most of these conditions, as is the fetal lethality of the homozygous state in rodents. Each of the genes is a tumor suppressor and encodes a protein that functions in signal transduction and is critical in mammalian development. Finally, for the majority of the genes, mutations are known to be common in nonhereditary cases of the corresponding tumors. Do these properties provide new criteria for classification of a disease as a phakomatosis?

The list of cloned phakomatosis genes constitutes about one third of the hereditary cancer genes that have been cloned. The tumors to which the phakomatoses predispose include several carcinomas and are generally quite different from the tumors featured in the conditions associated with mutations in the RB1, TP53, WT1, and CDKN2 tumor suppressor genes. Furthermore, benign precursors, such as adenomas and hamartomas, are clinically featured in the phakomatoses, again in distinction to the phenotypes observed with these other four genes. Mutations of the phakomatosis genes are apparently not directly transforming, but rather they act by producing increased numbers of dividing target cells.

Expression of the p27 protein, a CDK inhibitor, is depressed following mutation in phakomatosis genes NF1, VHL, TSC2, and PTEN, suggesting a special importance for p27. J. Letterio (NCI, Bethesda) pointed out that loss of expression of this gene is a bad prognostic factor in many cancers. Normally, the p27 protein inhibits cyclin D, E, and A complexes and increases growth-inhibitory signals, and the fraction of nonproliferative cells increases. TGFβ (transforming growth factor-β) protein is another well-known inhibitor of proliferation that activates p27. Mice heterozygous for mutation in or loss of p27 are larger than normal, and homozygously defective mice are still larger. The latter mice have a high incidence of pheochromocytoma, C-cell hyperplasia in the medulla of the thyroid, and tumors of the pars intermedia of the pituitary. Heterozygous mice exhibit increased susceptibility to tumors in the gut and lung in response to γ-irradiation and chemical carcinogens.

The phakomatoses were so categorized 67 years ago on the basis of clinical and pathologic features. Re-examining them in the light of the genes that have been cloned in the 1990s lends support to the classification because of the genes' common functional feature: being tumor suppressors involved in signal transduction. How remarkable it is that the phakomatoses have so much in common to the contemporary eye.

NOTES

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